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Acquired Resistance Induces Cross- and Pan-resistance to Some Chemotherapeutic Drugs in Breast Cancer Cell Lines

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ABSTRACT

Cancer is rapidly becoming a global pandemic. Even with the advancement in advancement in early detection of breast cancer andintroduction of new chemotherapeutic regimen. Chemoresistance is a feature of Cancer Stem cell and a major problem for chemotherapy.Primary and acquired resistance represents a major challenge for the effectiveness of chemotherapeutic agents in breast cancer management. This study was performed to evaluate the drug sensitivity of MDA-MB $231_{GEM100nM}$ and $T47D_{Dox100nM}$ to a number of conventional anticancer drugs and the possible mechanisms for drug resistance.Data from MTT analysis, western blot and fluorescence-activated cell sorting (*FASC*) indicated that the breast cancer cell lines exhibited cross resistance and pan-resistance to a number of chemotherapeutic drugs.

Key words: Pan-resistance, breast cancer cell line, cross-resistance, chemotherapy

INTRODUCTION

Cancers, one of the leading causes of death worldwide is rapidly becoming a global pandemic with cancer-related death exceeding death caused by stroke and coronary heart diseases (Ferlay *et al.,* 2013). The most frequently diagnosed carcinomas worldwide are lung, female breast cancer, colorectal and stomach cancers representing about 40% of cancer cases. Breast cancer (BC)is the second most common cancer in the world and the most fatal malignancy affecting women both in the developed and developing world. It is a major culprit associated with cancer-related deaths occurring in women worldwide(Weigelt and Reis-Filho, 2009).

Despite the advancement in early detection of breast cancer, a small proportion of women present with metastatic disease at the first time of presentation (Early Breast Cancer Trialists' Collaborative Group, 2005) or are prone to develop distant metastasis even though their initial diagnosis was non-metastatic breast cancer. Metastatic breast cancer though not curable has improved in survival due to the introduction of new chemotherapeutic regimen (Dafni *et al.,* 2010).

Chemotherapeutic drugs target cancer cells via circulation in blood by inhibiting cancer cell proliferation and inducing apoptosis in cancer cells. Rapidly dividing normal cells can also be affected by these cytotoxic drugs but are more likely to undergo repair. Anti-cancer drugs work by disrupting the growth of cancer cells. Chemotherapy is the standard care for patients with node-positive cancer and is of great benefit to patients with hormone receptor negative tumours (Goldhirsch *et al*., 2007).

Primary and acquired resistance represents a major challenge for the effectiveness of chemotherapeutic agents in breast cancer management. Doxorubicin (Dox) an anthracycline antibiotic is one of the most widely used chemotherapuetic agents for BC and is a prefered single agent as well as used in combination regimens for recurrent and metastatic BC (Gluck, 2005). Resistance to Dox can occur through several mechanisms such as decreased drug accumulation in which there is an increase drug efflux due to overexpression of multidrug resistance protein P-glycoprotein, alteration in drug targets proteins via cellular downregulation of topoisomerase II expression, cellular suppression of apoptosis by upregulation of Glutathione-S-transferase (GST)and by enhanced capacity of drug-induced DNA repair (Tubbs *et al.,* 2009).

Gemcitabine (dFdC) can be used as a single agent for advanced BC or in combination therapy with other chemotherapeutic drugs such as cisplastin (CDDP) and paclitaxol (PTX) with even higher response rates. It is a first line drug for the treatment of BC and has a high initial activity against tumours but is prone to induce acquired resistance in cancer cells (Mini *et al*., 2006).

Many current cancer therapies are limited by the severity and frequency of adverse side effects associated with such treatments which has led to a search and high demand for non-toxic alternatives. One source of new therapies may be

through repurposing of clinically approved drugs, where safety in patients has already been established. Disulfiram (DS) an anti-alcoholism drug used in clinic for over 60 years (Eneanya *et al*., 1981) demonstrates anti-cancer effects in cancer cell lines (Cen *et al*., 2004) as well as in a range of solid and haematological malignancies (Wickstrom*et al.,* 2007). The clinical use of DS over a long period of time has shown it to be a very safe drug with minimal and manageable toxic effects, even at fairly high doses of 300 to 500 milligrams daily (Sauna *et al*., 2005). The biological activity of disulfiram is attributed to its ability to bind divalent cations and consequently disrupt metal dependent processes, principally those involving copper (Cu) and zinc (Zn) (Shiah *et. al*., 2003). Several *in vitro* experiments indicate that the cytotoxicity of DS is entirely dependent on copper(II) (Cu) or some other transition bivalent metal ions in the culture medium (Liu *et. al*., 2012; Tawari*et. al*., 2015).

Acquired resistance of initially senstive cancer cells to chemotherapeutic drugs is a major challenge for successful treatment of BC cells. The study was performed to evaluate the drug sensitivity of MDA-MB $231_{GEM100nM}$ and $T47D_{Dox100nM}$ to a number of conventional anticancer drugs and the possible mechanisms for drug resistance.

METHODOLOGY:

Cell lines and reagents:

Parental cell lineMDA-MB-231 and T47D were purchased from ATCC, Middlesex, UK and the resistant cell line MDA-MB-$231_{GEM100nM}$ and $T47D_{Dox100nM}$ were generated from the parental cell lines by continuously cultured in medium containing Gemcitabine(dFdC), and Doxorubicin (Dox) respectively (Sigma, Dorset, UK) in a stepwise concentration-increasing procedure.Gemcitabine, Paclitaxel (PTX), Cisplatin (CDDP), Vincristine (VCR), Doxorubicin, Disulfiram (DS)and copper (II) chloride ($CuCl_2$) were purchased from Sigma.

Cell culture and cytotoxicity analysis: All cell lines werecultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Wokingham, UK) supplementedwith 10% FCS, 50 unitsml_1 penicillin and 50 mgml_1 streptomycin. The MDA-MB-$231_{GEM100nM}$cells were maintained in the mediumcontaining 100nMof GEM and $T47D_{Dox100nM}$ cells were maintained in the mediumcontaining 100nM of DOX.

For in vitro cytotoxicity assay, the overnight cultured cells (5000 per well) in 96-well flat-bottomed microtiter plates were exposed to drugs for 72 hours (PAC),

dFdC 72hours, Vincristine (VCR) 48hours, DOX 72hours and 120 hours(CDDP) and subjected to a standard MTT assay (Plumb et al, 1989).

Western blotting analysis: The protein expression levels were determined by staining with primary antibodies and relevant HRP conjugated secondary antibodies.Primary antibodies picoglycoprotein (pgp) supplied by Santa Cruz, Dallas, TX, USA.

Detection of ALDH positive population

The Aldehyde dehydrogenase (ALDH) positive population was detected by ALDEFLUOR kit (StemCell Tech., Durham, NC, USA) following the supplier's instruction. The cells (2.5 × 105) were analyzed after stained in ALDH substrate containing assay buffer for 30 min at 37°C. The negative control was treated with diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor.

Flow cytometric analysis of CD 133

The adherent were trypsinised and passed through a 25G needle. The cells (2.5 × 105) were incubated with CD 133 antibody (BD Pharmingen, Oxford, UK) for 20 min at 4°C. Unbound antibodies were washed off with 2% fetal calf serum (FCS) HBSS (Sigma) and the cells (10,000 events) were examined no longer than 1 hour after staining on a BD Facscalibur.

Immunofluorescent flow cytometric analysis of embryonic stem cell markers

The expression of Nanog, Oct4 and Sox2 was determined by immunofluoresent flow cytometry. The cultured cells were collected by trypsinization. The cells fixed by acetone/methanol and permeabilized by 0.1% triton-X100. After blocked with 3% BSA for 1 hour the cells were stained with primary (1:50 dilution) and FITC-conjugated secondary antibodies respectively for 1 hour at RT. The positively stained population was detected using a FACSCalibur flow cytometer with 488-nm blue laser and standard FITC 530/30 nm bandpass filter.

Statistical Analysis

SPSS 13.0 Student's *t* test and one way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test were used to calculate the differences. Data were expressed as mean±SD. $P \leq 0.05$ was considered as significantly change.

Results

Morphological features

The drug resistance cells were observed to have a different phenotype than the wild type (WT) parental cells. Figure 1 shows that the resistant cells were smaller and with less defined irregular multiple nucleiwhen compared to the parental cell lines.

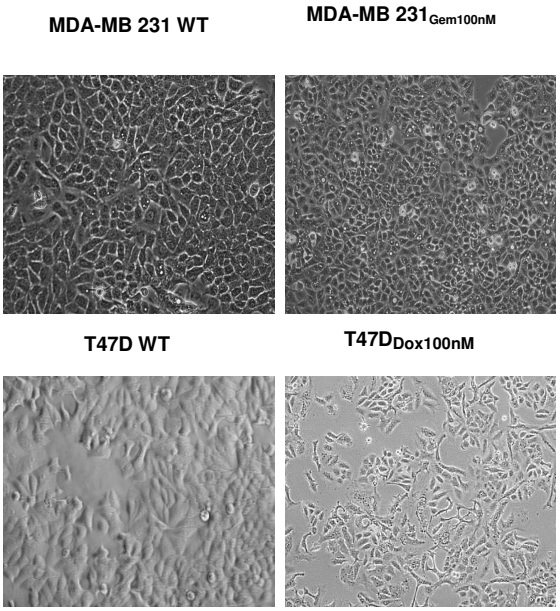


Figure 1: Representative Images of Morphology of wild type and resistant cells.
Images taken by light microscopyshows themorphology of parental and resistant cells (Magnification 20X) after culturing in normoxia. (Abbreviations WT: wide type; MDA-MB-231$_{GEM100}$nM: MDA-MB231 gemcitabine resistant cell, T47D $_{Dox100nM}$:T47D doxorubicin resistant cell).

Resistant cell lines show cross and pan-resistance to anticancer drugs

Data from MTT cytotoxicity analysis showed that apart from being highly resistant to dFdC (Figure 2), the MDA-MB 231$_{Gem100nM}$ cell line is also cross resistant to other anticancer drugs e.g. CDDP, Dox, VCR and PTX (Figure 3). The parental cell line has a much lower IC$_{50s}$ compared to that of the resistant cell line MDA-MB 231$_{Gem100nM}$(Table 1).

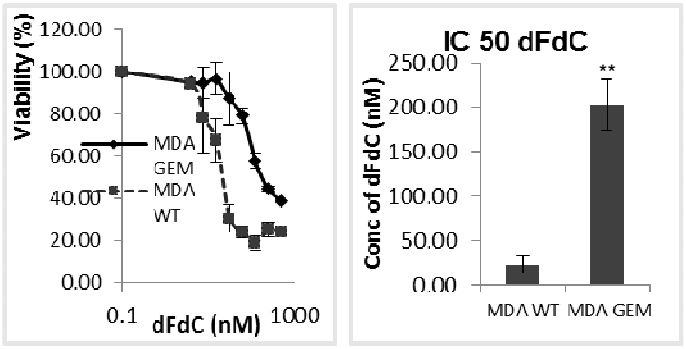


Figure 2: Representative Drug Concentration Response Curve of MDA-MB 231 and MDA-MB 231$_{Gem100nM}$ cell line.**p=<0.001, n=9 (Abbreviations; MDA-WT: MDA-MB 231 wild type cell line, MDA GEM: MDA-MB 231 gemcitabine resistant cell line, dFdC: gemcitabine).

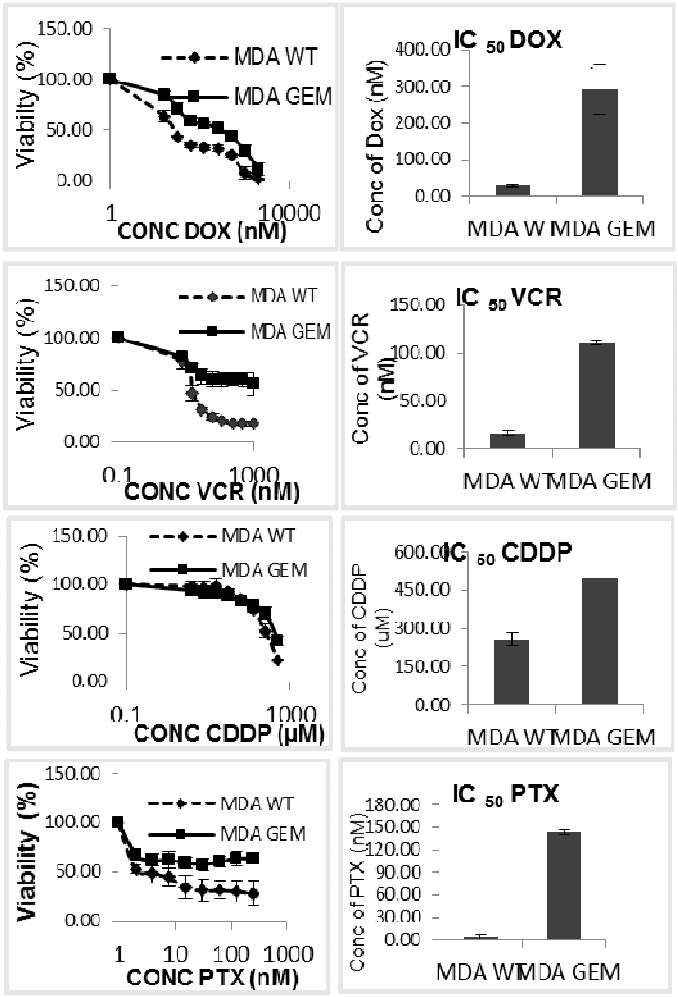


Figure 3. Representative Drug Concentration Response Curves of MDA-MB 231 and MDA-MB 231$_{Gem100nM}$ cell line.(gemcitabine resistance cell line, VCR: vincristine, Dox: doxorubicin, CDDP: cisplatin and PTX: paclitaxel).

Anti-cancer drug/ IC_{50}	MDA-MB 231 WT	MDA-MB231 GEM100nM	U-value	p-value
Cisplatin (µM)	650.01 ±5.89	> 1500	24.19	0.0004**
Paclitaxel (nM)	4.01± 0.11	> 500	32.15	0.0001**
Vincristine (nM)	13.22 ± 1.06	> 900	31.97	0.0001**
Gemcitabine (nM)	22.17 ± 6.84	> 800	23.45	0.0001**
Doxorubicin (nM)	50 ± 108.73	> 500	29.32	0.0001**

Table 1: IC_{50} Value from cell viability curve in Resistant Cell Line MDA-MB 231$_{GEM\ 100nM}$ and Sensitive Cell Line MDA-MB231 WT

Similar results were also obtained for the T47D$_{Dox100nM}$ cell line, in which resistance to Dox was observed and cross resistance to other anticancer drug with various mechanism of action (Figure 4 A and B). Cell viability curve from MTT analysis shows difference in dose response between resistant cell (T47D$_{Dox100nM}$) and parental cell (T47D WT). The resistant cell displayed significant resistance to Dox and displayed significant cross resistance to anticancer drugs (VCR, dFdC, CDDP and PTX). Histogram (median ± interquartile range) shows elevated half maximal inhibitory concentration (IC_{50}) values indicating increased drug resistance to doxorubicin (Mann and Whitney U test, resistance, **p=<0.001, n=9).

Figure 4A. Representative Drug Concentration Response Curve of T47D and T47D$_{Dox100nM}$ cell line.(Abbreviations; T47D-WT: T47D wild type cell line, T47D DOX: T47D doxorubicin resistant cell line, Dox: doxorubicin).

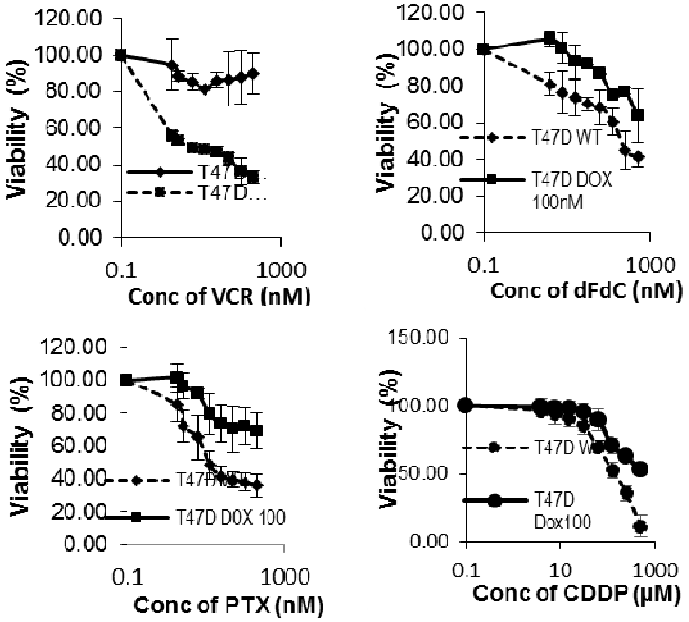
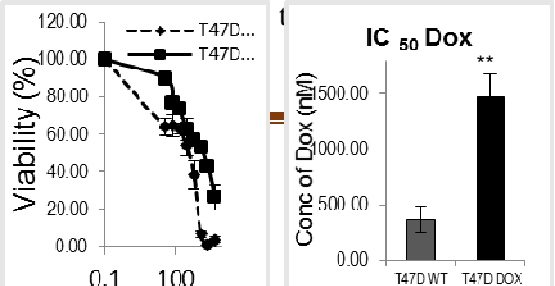


Figure 4B: Representative Drug Concentration Response Curves of T47D and T47D$_{Dox100nM}$ cell line to conventional anticancer drugs.

Table 1 displays the results (median ± interquartile range) of half maximum inhibitory concentration (IC_{50}) valuesofT47D wild type and T47D$_{Dox\ 100nM}$ resistant cell line. The resistant cell line T47D



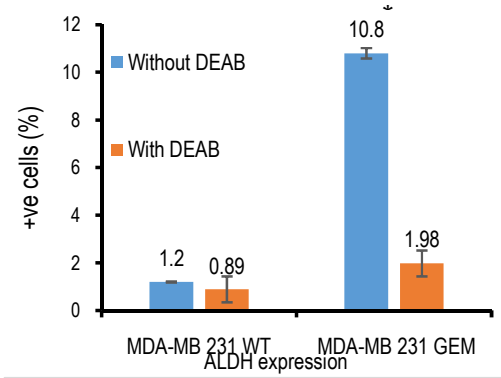
IC_{50} and were significantly resistant to these conventional anticancer drugs.

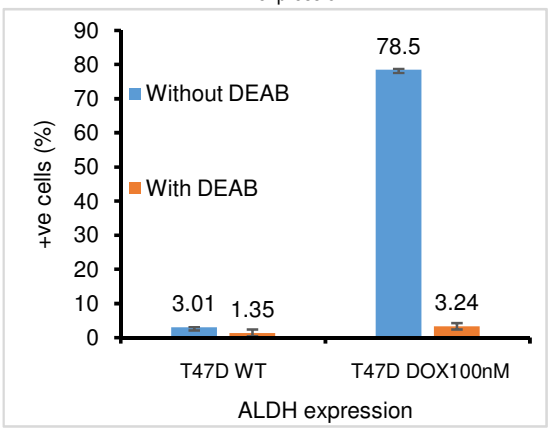
Anti-cancer drug/ IC_{50}	T47D WT	T47D $_{Dox100nM}$	U-value	p-value
Cisplatin (µM)	198.01 ± 5.89	>600	17.12	0.0002**
Paclitaxel (nM)	7.69± 0.26	> 200	36.29	0.0001**
Vincristine (nM)	4.81 ± 3.56	> 250	31.97	0.0001**
Gemcitabine (nM)	224.54 ± 4.81	> 600	23.45	0.0001**
Doxorubicin (nM)	365.78 ± 108.73	> 1000	29.32	0.0001**

Table 2: IC_{50} Value from cell viability curve in Resistant Cell Line $T47D_{Dox\ 100nM}$ and Sensitive Cell Line T47D WT

Resistant cell lines displayed an increase in CSC markers

FASC results (Figure 5)shows Aldehyde dehydrogenase (ALDH) activity in the parental cell line (MDA-MB 231 WT and T47D WT)and resistant cell lines (MDA-MB $231_{GEM100nM}$and $T47D_{Dox100nM}$) with and without treatment with DEAB (30µM). The resistant cell lines expressed high percentage of $ALDH^+$ compared to the parental cells before treatment with Diethylaminobenzaldehyde (DEAB). Histogram (median ± interquartile range) displays statistically significant increase in the $ALDH^+$ activity in resistant cell lines in comparison to parental cell lines(Mann and Whitney U test, ***p=<0.0001, n=9*). After treatment with DEAB, there was no statistical difference in ALDH+ cell from both cultures (p>0.05).





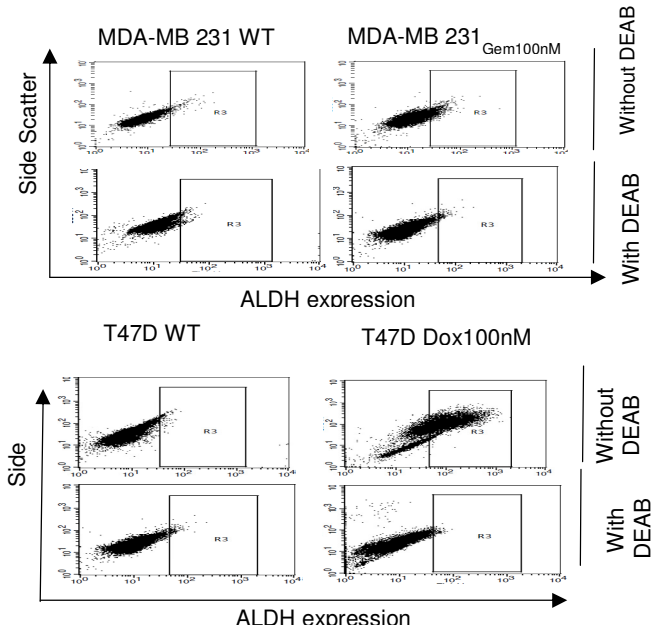


Figure 5:Representative FASCS Plots and Histograms of ALDH expression in MDA-MB 231 WT and MDA-MB $231_{GEM100nM}$and T47D WT and $T47D_{Dox100nM}$cell linemeasured by ALDEFLUOR assay.

FASC data (Figure 6A and B) shows CD133 expression in in the parental cell line (MDA-MB 231 WT and T47D WT) and resistant cell lines (MDA-MB $231_{GEM100nM}$and $T47D_{Dox100nM}$).There was an increase in the expression of CD133 in the resistant cell lines compared to parental cell line. Histograms (median ± interquartile range) displays the statistically significant increase in the CD133 expression in resistant cells in comparison to sensitive cell line (Mann and Whitney U test, ***p=<0.0001, n=9)*

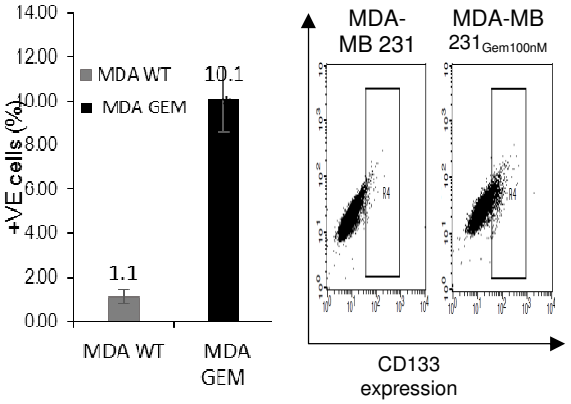


Figure 6 A. Representative FASC Plots of CD133 expression in MDA-MB 231 WT and MDA-MB 231GEM100nM BC cell line by PE-CD133 immunostaining assay.

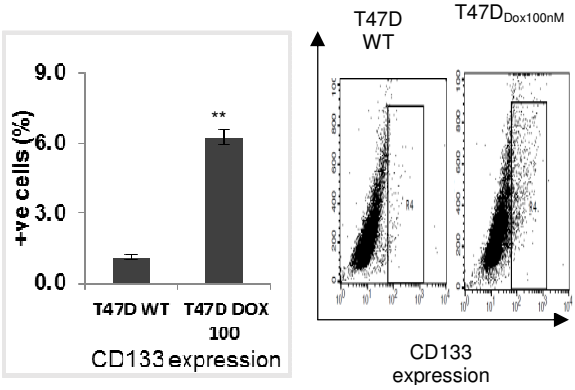


Figure 6B: Representative FASC Plots of CD133 expression in T47D WT and $T47D_{Dox100nM}$ BC cell line measured by PE-CD133 immunostaining assay.

Resistant cell lines displayed an increase in embryonic stem cell markers

The embryonic stem cells (Nanog, Sox2, Oct4) important for maintenance of stem cell, renewal and pluripotency were also significantly increased in the resistant cell lines MDA-MB-$231_{GEM100nM}$ and $T47D_{Dox100nM}$ in comparison to the parental cell MDA-MB-231and T47D respectively.*Fluorescence Activated Cell Sorting* (FASC) data (Figure 7 (A)(B))shows that higher expression of embryonic stem cell markers (nanog, Sox2 and Oct4) were higher in resistant cell lines (MDA-MB-$231_{GEM100nM}$ and $T47D_{Dox100nM}$)compared to parental cell MDA-MB 231 and T47D respectively.

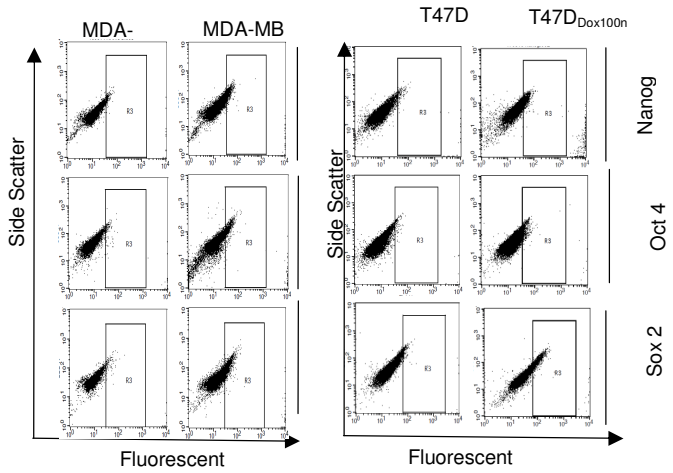


Figure 7(A). Representative FASC Plots of Embryonic Stem cell markers expression in in sensitive cell lines (MDA-MB 231 WT; T47D WT) and resistant cell lines (MDA-MB 231$_{GEM100nM;}$ and T47D$_{Dox100nM}$).

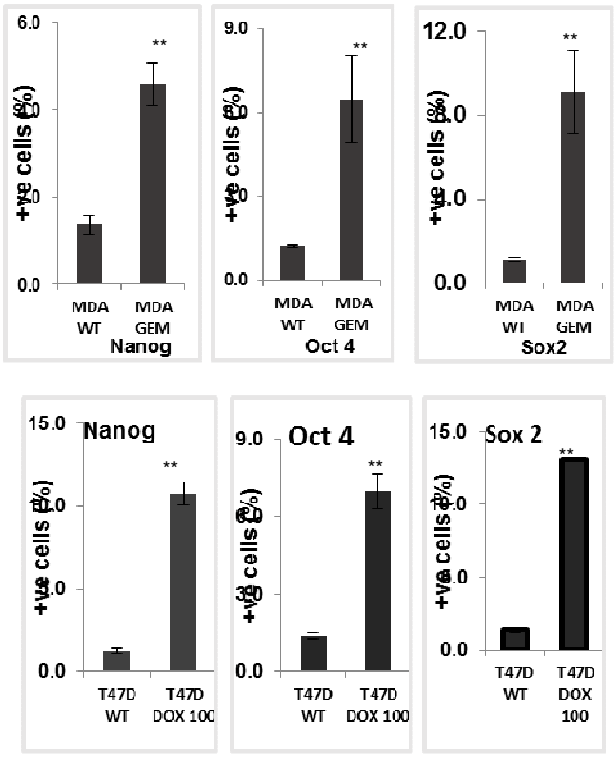


Figure 7B Bar chart Representation of embryonic stem cell markers expression in sensitive cell lines (MDA-MB 231 WT; T47D WT) and resistant cell lines (MDA-MB 231$_{GEM100nM;}$ and T47D$_{Dox100nM}$).

Resistant cell lines have longer doubling time

Quiescence is a common feature of drug resistant cancer cell lines. The cell growth of resistant cells MDA-MB-231$_{GEM100nM}$ and T47D$_{Dox100nM}$ were significantly slower compared to the parental wide type cells MDA-MB-231 and T47D respectively. Figure 8 shows the growth curve (Mean ± SD) for wide type cells and resistant cells (MDA-MB-231$_{GEM100nM}$and T47D$_{DOX100nM}$ cell lines) proliferates slower than their respective wide type cells indicating that the resistant cells have a longer doubling time.

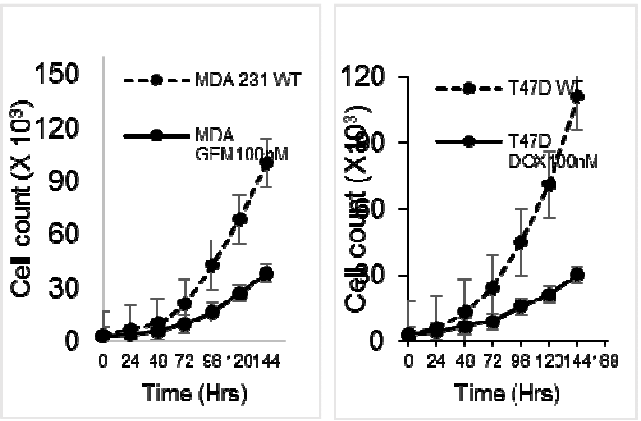


Figure 8. Representative Growth curves of MDA-MB-231 and MDA-MB-231$_{GEM100nM}$ cells and T47D and T47D$_{DOX100nM}$ cell lines.

Western blot analysis of antiapoptotic proteins

Western blot analysis performed on whole cell lysate of $T47D_{Dox100nM}$ resistant cell line show that expression of P-gp may be a major factor contributing to drug resistance in these cells.

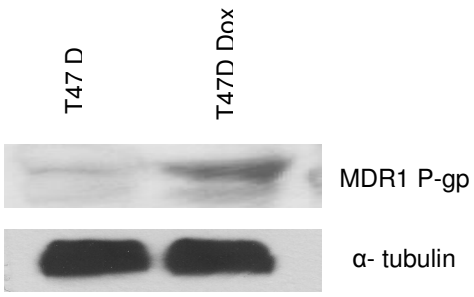


Figure 9. Representative Western Blot of MDR1 P-pg expression in T47D wild type and $T47D_{Dox100nM}$.

Resistant cells exhibit reduce cytotoxic apoptotic induced death

Several morphological changes were observed in the resistant cell lines. Images captured using light microscope show that the MDA-MB $231_{GEM100nM}$ cells were smaller than the parental cell MDA-MB 231 cells. The $T47D_{Dox100nM}$ appeared to have multiple nuclei, spindle like with elongated pseudopodia and have a less cohesive appearance while parental T47D cells have a cobblestone appearance with single nuclei.

Cell death was observed in the parental cells MDA-MB 231 and T47D cells after exposure to 200nM and 700nM of dFdC and Dox respectively, while the resistant cells were tolerant to the anticancer drugs. Treatment of cells with Ds/Cu induced massive cell death. Images (Figure 10 A and B) showed the morphology (× 20 magnification) of resistant cell lines ($MDA\text{-}MB\text{-}231_{GEM100nM}$ and $T47D_{Dox100nM}$) and parental cell (MDA-MB 231 and T47D) before and after treatment with dFdC (200nM) and Dox(700nM). After 72 hours, cell death was observed in the MDA-MB 231 treated parental cells while the resistant cells survived. Treatment of MDA-231 WT and $MDA\text{-}MB231_{GEM100nM}$ cell lines with DS plus Cu for 4 hours induced cell death in both resistant and sensitive cell lines.

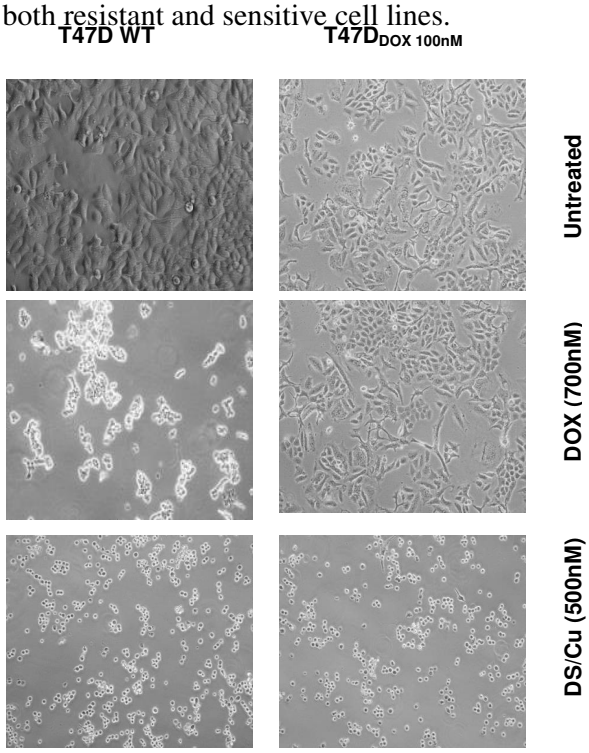


Figure 10A: Representative Images of sensitive cell lines (T47D WT) and resistant cell lines (and

$T47D_{Dox100nM}$).(Abbreviations: WT: wild type, **$T47D_{Dox100nM}$**: Dox resistance cell, DS: disulfiram, Cu: copper).

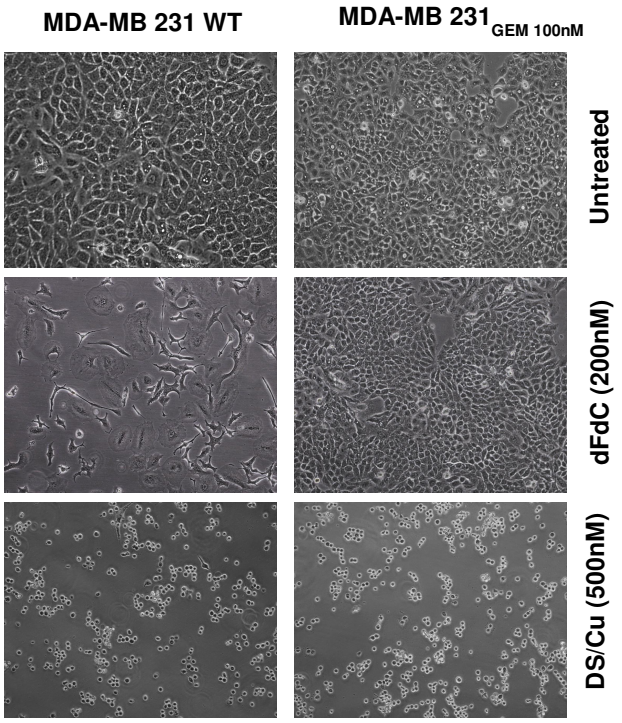


Figure 10. Representative Images of sensitive cell lines (MDA-MB 231 WT) and resistant cell lines (MDA-MB $231_{GEM100nM}$).

(Abbreviations: dFdC: gemcitabine, WT: wild type, GEM100nM: gemcitabine resistance cell, DS: disulfiram, Cu: copper).

Discussion

Chemotherapy the use of cytotoxic drugs is one of the standard methods of treatment for many cancers. Chemotherapy induces cell death in tumours reducing the tumour bulk however many patients experience tumour recurrence and ultimately death. Resistance to these therapeutic drugs remains a major hurdle in the treatment and management of BC. In this study, two resistant cell lines MDA-MB-$231_{GEM100nM}$ and $T47D_{Dox100nM}$ were characterized for better understanding on the mechanisms of chemo resistance involved in resistance to dFdC and Dox respectively.

In vitro cytotoxicity assay showed that MDA-MB-$231_{GEM100nM}$ cell lines were significantly resistant to dFdC-induced cytotoxicity compared to the sensitive parental MDA-MB 231 cell line. The resistant cell line was also cross resistant to four other conventional anticancer drugs (CDDP, VCR, PTX, Dox) all with different mechanism of action (Figure 4A and B), indicative that acquired resistance to dFdC can induce pan-resistance to a range of anticancer drugs which is in agreement with the report by Faneyte *et al.,* (2001) which demonstrated that BC cells initially responsive to anticancer drugs frequently relapsed and acquired resistance to a broad spectrum of drugs.

Previous studies(Chen *et al.,* 2008; Zhang *et al.,* 2009) show that CSCs play vital roles in the induction of chemoresistance in BC cells. To further understand the mechanism of resistance of dFdC, flow cytometry assay was performed in the MDA-MB 231parental cell and MDA-MB $231_{GEM100nM}$ cell line. The resistant cell line MDA-MB $231_{GEM100nM}$ had higher expression of CSC markers suggestive that these cells had a

stem-like phenotype which may be the cause for the chemoresistance.

Doxorubicin (Dox) an anthracycline antibiotic is considered to be one of the most effective agent in the treatment of breast cancer, however resistance to Dox leads to unsuccessful outcome in many patient. MTT cytotoxicity analysis showed that the $T47D_{Dox100nM}$ resistant cells were also found to be resistance to Dox and other conventional anticancer drugs. Similar to the findings in dFdC resistant cells, the Dox resistant cells also displayed CSCs like phenotype as stem cell markers ALDH, CD 133 and embryonic markers (Nanog, Sox2 and Oct4) were all significantly increased in $T47D_{Dox100nM}$ cells compared to the parental T47Dwild type cell. Further confirming the involvement of stem cells in drug resistance.

Another mechanism of resistance in cancer cell is overexpression of multi drug resistance (MDR) related ATP-binding cassette(ABC) transporters which increase drug efflux from cells. Dox resistance is predominantly due to the expression of P-gp (Broxterman *et al.,* 1995).P-gp a multidrug efflux pump responsible for drug resistant in a number of cancer cancers was also slightly upregulated in the T47D $_{Dox100nM}$ resistant cell line but was not detected in MDA-MB-$231_{GEM100nM}$ cells, suggesting that P-gp could be responsible for the resistance in Dox resistant cells but was not the case for the dFdC resistant cells.

Conclusion

These resistant cell lines could be useful model to investigate the mechanisms of resistance in BC cells. This study shows that the resistant cells were highly resistant to a range of anticancer drugs suggestive that acquired resistance can induce pan-resistance in BC cells. The resistant cells had slow proliferation rate as shown in the growth curve displaying senescence. We believe that the induction of stem-like cell features (increased stem cell markers) in these cells together with the several antiapoptotic genes were responsible for drug resistance, therefore development of drugs that can target these CSCs would be beneficial.

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